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In vitro investigation of autoantibody-secreting peritoneal cells and their regulation

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Summary. A high proportion of peritoneal cells from untreated mice, after 4–5 days in culture, develop into plaque-forming cells against bromelain-treated mouse red blood cells. The number of plaque-forming cells was increased significantly by exposing the peritoneal cells to ammonium chloride to lyse red blood cells before culture. Conversely, the increase was significantly inhibited by adding before culture untreated or bromelain-treated sheep or mouse red blood cells. Treated or untreated horse or rat red blood cells did not inhibit the increase. Treating peritoneal cells or subpopulations of peritoneal cells with anti-θ serum and complement before culture caused a significant increase in the number of plaque-forming cells against bromelain-treated red blood cells after 3–4 days of culture. Various procedures were used to fractionate peritoneal cells into B-cell enriched and B-cell depleted subpopulations before culture and after culture, to investigate whether some of the plaque-forming cells could be attributed to phagocytic cells. Generally, changes in the number of plaque-forming cells against bromelain-treated mouse red blood cells paralleled changes in B-cells. In some experiments the proportion of plaque-forming cells observed represented up to 85% of the B-cells present. The results suggest that the high level of autoreactivity is due to antibody production by B-cells and that phagocytic cells are not forming spurious plaques. Further, it appears that the autoimmunity is regulated by T-cells and can also be inhibited by mouse RBC.

Abbreviations: brom, bromelain-treated; PFC, plaque-forming cell; RBC, red blood cell; Ig, immunoglobulin; FCS, foetal calf serum; PBSG, phosphate-buffered saline and glucose; SIg^+, surface immunoglobulin; LPS, lipopolysaccharide.

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INTRODUCTION

Various lymphoid tissues of unimmunized mice, including germ-free mice, contain a high background number of cells secreting antibodies directed against mouse enzyme-modified red blood cells (RBC) (reviewed by Cunningham, 1976). The proteolytic modification, with enzymes such as bromelain, removes the major external protein and some of the glycoproteins from the RBC membrane and exposes 'internal' antigens (Cunliffe & Cox, 1979). The number of plaque-forming cells (PFC) against mouse bromelain-treated (brom) RBC is not increased by injections of modified RBC but is increased significantly by injecting mice with anti-lymphocyte serum or lipopolysaccharide. From these observations it was suggested that normally this autoimmune response is strongly controlled, possibly by suppressor T-cells (Cunningham, 1976). Using conventional techniques,

delayed-type hypersensitivity against mouse brom RBC was not observed (Cox, Baddams & Evans, 1977), although spleen cells cultured *in vitro* for 4 days produced delayed-type hypersensitivity against mouse brom RBC upon injection into syngeneic mice (Ramshaw & Eidinger, 1977).

Earlier observations showed that very high numbers of PFC against sheep RBC were detected after culturing peritoneal cells *in vitro* for 4 days (Bussard, 1966). Various procedures suggested that this unusually high response, which occurred in cells from unimmunized mice without adding sheep RBC to the cultures, was due to specific antibody production (Gisler, Pages & Bussard, 1975). More recently it has been noted that sheep RBC, sheep brom RBC and mouse brom RBC share some common antigens (Pages & Bussard, 1975) and that culture of peritoneal cells *in vitro* leads to a big increase, after about 4 days, in the number of PFC against mouse brom RBC (Pages & Bussard, 1975; Lord & Dutton, 1975a). Although the peritoneal cells did not undergo DNA synthesis in culture, the PFC were shown to be due to release of antibodies because plaque formation required protein synthesis and was prevented by adding anti-mouse Ig sera to the assay mixtures (Lord & Dutton 1975b). Culturing the peritoneal cells with mouse brom RBC inhibited the autoimmune response *in vitro* (Lord & Dutton 1975b).

The specificity of these PFC against mouse brom RBC has been confirmed by inhibiting plaque formation with a soluble antigen from mouse plasma (DeHeer & Edgington, 1974) and with stromata from mouse brom RBC (Bussard, Vinit & Pages, 1977). The latter group also showed that individual PFC could be micromanipulated to form more than one plaque, which suggested that plaque formation was not due to release of passively acquired antibody.

Steele & Cunningham (1978) have shown that in peritoneal cell cultures, a high proportion of all Ig-secreting cells are specific for mouse brom RBC. They showed that removing the cells that formed rosettes with mouse brom RBC before culture abolished the appearance of PFC against mouse brom RBC.

We have used various procedures to deplete or enrich peritoneal cells of B cells, both before and after *in vitro* culture, to assess whether some of the PFC against mouse brom RBC were due to cells other than B cells. It was shown that B cells produced the plaques. It was also shown that antibody production against mouse brom RBC was inhibited by T cells and, separately, by mouse RBC or mouse brom RBC.

MATERIALS AND METHODS

Mice

Inbred C3H mice were purchased from the Institute of Medical and Veterinary Sciences (IMVS) in Adelaide. Unimmunized mice of either sex, aged between 6 and 12 weeks were used.

RBC suspensions

Sterile sheep RBC and horse RBC in Alsever's solution were obtained from the IMVS and used within 2 weeks. Rat RBC and mouse RBC were collected into Alsever's solution from our animal stocks and used on the day of collection. The method used for bromelain modification of RBC has been given (Cox *et al.*, 1977)

Collection of peritoneal cells

Five millilitres of RPMI 1640 (Flow Laboratories, Sydney) supplemented with 1% foetal calf serum (FCS) and 1% of 200 mmol glutamine was injected into the peritoneal cavity of each mouse. The abdomen was massaged for 30 s and the medium withdrawn. Usually about 4 ml containing 1×10^6 to 3×10^6 viable nucleated cells were recovered. The cells from three mice were pooled and centrifuged at 400 *g* for 7 min at 4°. Unless stated otherwise, all cell suspensions were subjected to a wash with ammonium chloride to lyse contaminating RBC. Thus, the cells in the pellet after centrifugation were resuspended in 3 ml of an ice-cold solution of 0·184 M ammonium chloride and underlaid with 1 ml of FCS. After 5 min on ice the suspensions were centrifuged as before, and the cells were recovered and washed three times in 15 ml of culture medium. All cell suspensions within the same group of mice were pooled, and clumps were removed by centrifuging at 50 *g* for 30 s. The number of viable nucleated cells was estimated by trypan blue exclusion.

Cell culture

1×10^5 viable cells per 0·3 ml per well were cultured in Linbro flat-bottom tissue-culture plates (6 mm, Flow Laboratories, Sydney). The culture medium was RPMI 1640 supplemented with 10% FCS and 1% 200 mmol L-glutamine and 2-mercaptoethanol at a final concentration of 5×10^{-5} M. Throughout the culture period the cells were maintained in an atmosphere of 5% CO_2/95% air at 37°. In some experiments RBC suspensions were added at a final concentration in each well of 5×10^6 RBC/ml. The RBC suspensions

were mixed with the peritoneal cells before culture and 0·3 ml per well of the mixture was transferred to the culture plates.

Harvesting cells following culture
Unless stated otherwise, the cells were recovered by gentle aspiration and by scraping the bottom of the wells using a pipette. The cells from six wells were pooled, made up to 15 ml with culture medium and centrifuged as before. Routinely there were at least eighteen wells per day for each particular type of cell suspension. After centrifugation, the cells were resuspended in 0·6 ml medium. The number of PFC, viable cells, phagocytic cells and cells bearing surface Ig (SIg^+) were estimated. To estimate PFC against mouse brom RBC, duplicate 0·1 ml aliquots were assayed.

Assay for PFC against mouse brom RBC
The procedure has been described (Cox *et al.*, 1977). The PFC were inhibited by including in the assay mixture an antiserum against mouse μ chains (Bionetics, Maryland, U.S.A.) at a concentration that did not inhibit IgG PFC in a secondary response to sheep RBC. An antiserum against mouse γ chains did not inhibit PFC against mouse brom RBC. Unless stated otherwise, the PFC detected have been expressed as the mean number per 10^6 viable cells cultured on day 0 for triplicate cultures (cells from six wells pooled for each culture) $\pm$ one standard deviation.

Detection of surface Ig-bearing cells
A fluorescein conjugated goat anti-mouse Ig serum (Behringwerke, Germany) was used Between 100 and 300 cells for each cell suspension were examined for fluorescence. Thymus cells did not fluoresce whereas about 30% of spleen cell suspensions were fluorescent. The mean number of fluorescent cells detected in triplicate cultures was expressed as a percentage of all cells examined.

Detection of phagocytic cells
The method described by DeHeer & Edgington (1976) was used to detect phagocytic cells. At least 100 cells were examined and those with three or more particles of iron dextran were classified as phagocytic. The mean number of phagocytic cells detected in triplicate cultures was expressed as a percentage of all cells examined.

Separation of adherent and non-adherent cells
These were separated according to the method of Rosenberg & Parish (1977). Peritoneal cells, before being set up in the usual protocol, were incubated in plastic petri dishes (60 × 15 mm Kayline Plastics, Adelaide) for 4 h at 37° in culture medium. Non-adherent cells were recovered by removing culture medium and gently washing the dishes three times with culture medium, each wash being 3 ml. Adherent cells were dislodged with a 'rubber policeman' and taken up in culture medium. Both cell suspensions were washed once in culture medium. For control cultures, adherent and non-adherent cells were recovered as indicated and pooled again prior to culture.

After culture, non-adherent cells were taken up by very gentle aspiration of the culture medium. The adherent cells were removed by adding another 0·3 ml of medium and aspirating vigorously. The harvested cells were washed once in 15 ml of medium.

Enrichment of B-cells using sheep RBC rosettes
The method described by Parish, Kirov, Bowern & Blanden (1974) was used. For control cultures, the cells recovered from the pellet and the cells from the interface were mixed.

Enrichment of B cells using Ig-coated dishes
The method of Wysocki & Sato (1978) was followed. Plastic petri dishes (60 × 15 mm, Kayline, Adelaide) were coated with a mixture of rabbit anti-mouse gammaglobulin (ammonium sulphate precipitated) diluted 1 : 9 with normal rabbit gammaglobulin. This mixture was diluted to a concentration of 10 μg/ml in 0·04 M Tris buffer, (pH 9·5), and 10 ml poured onto the plates. The solution was swirled until each plate was evenly covered. After 40 min at 15–20°, the buffer was decanted and the dishes washed four times in PBSG and once in culture medium. A 3 ml aliquot of washed peritoneal cells (2×10^6 cells/ml) was poured onto each plate. The plates were kept on ice for 7 min. After 40 min, unattached cells were distributed by swirling the plates. At the end of a further 30 min, the non-adherent cells were decanted and the plates washed twice with culture medium. Bound cells, containing a B-cell enriched fraction, were recovered by adding 15 ml of culture medium to each plate and scraping the bottom with a pipette. Both suspensions of cells were centrifuged and viable cells estimated as before. For control cultures, the adherent and non-adherent cell suspensions were pooled.

Anti-θ treatment of cells

Washed peritoneal cells were adjusted to a concentration of 2×10^6 cells/ml and an equal volume of a 1 in 5 dilution of anti-θ serum (Bionetics, Maryland, U.S.A.) in PBSG was added to the cell suspension. This mixture was held at 4° for 60 min. Guinea-pig serum, which had been absorbed twice with mouse liver cells, was added to a final concentration of 1 in 20 and the mixture incubated for 60 min at 37° in a 5% CO_2/95% air atomsphere. Following incubation, the cells were washed once in culture medium and viable cells estimated as before. In control cultures PBSG was used in place of anti-θ serum.

Statistical analysis

Two-tailed Student's *t* tests were used to determine whether differences between means were significant. A probability level of 0·05, or less, was accepted as a significant difference.

RESULTS

Peritoneal cells from unimmunized C3H mice were assayed at daily intervals during *in vitro* culture for the number of cells secreting antibodies against mouse brom RBC. Before culture, and after 1 and 2 days of culture, a low number of PFC was detected. The number rose significantly during the next 2–3 days of culture often reaching up to 10^4 PFC/10^6 viable cells cultured (Fig. 1)

Pre-treating the peritoneal cells with ammonium chloride to remove mouse RBC prior to culture caused the PFC to appear earlier and in higher numbers (Fig. 1).

The number of PFC reacting against mouse brom RBC was significantly reduced by culturing mixtures of peritoneal cells and either syngeneic mouse RBC or mouse brom RBC (Fig. 2). This suppression was also caused by sheep RBC or sheep brom RBC but not by bromelain-treated, or untreated, horse or rat RBC (Fig. 2).

The peritoneal cells were characterized before culture and after 4 days of culture to determine whether the culture conditions had selected particular subpopulations of cells. The proportion of B cells and phagocytic cells detected after 4 days of culture was not significantly different from that detected before culture (Table 1).

Various procedures were used to fractionate the peritoneal cell population, either before or after *in vitro* culture, into subpopulations enriched for B cells or phagocytic cells. In the first technique, peritoneal cells were separated before culture, using adherence to plastic, into adherent cells (mainly macrophages) and non-adherent cells (mainly B cells) and a control population in which the adherent and non-adherent cells were mixed again after separation (Table 2). After 4 days in culture, the number of PFC against mouse brom RBC was increased in the B-cell enriched cultures and decreased in the B-cell depleted cultures, although not on a *pro rata* basis (Table 2). The proportion of B cells and phagocytic cells was similar before and after 4 days of culture (Table 2).

In other experiments non-adherent cells were recovered after *in vitro* culture for 4 days before assaying for PFC against mouse brom RBC. About 30% of these cells formed plaques on monolayers of mouse brom RBC although only 35% were SIg^+ (Table 3). Simple arithmetic shows that 83% of the peritoneal B-cell population were secreting antibodies that lysed mouse brom RBC. In the non-adherent cell subpopulation, a lower number of PFC was paralleled with a

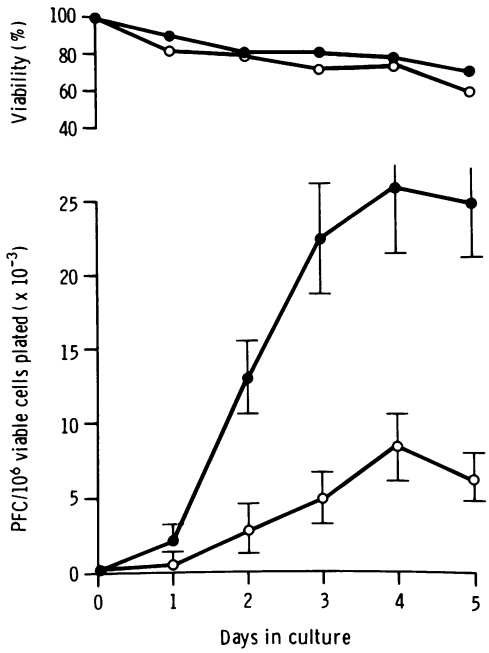

Figure 1. The number of PFC against mouse brom RBC and the proportion of viable cells after various periods of *in vitro* culture of peritoneal cells. The points are mean values for triplicate cultures ± one standard deviation. •, Cells treated with ammonium chloride prior to culture; o, untreated cells.

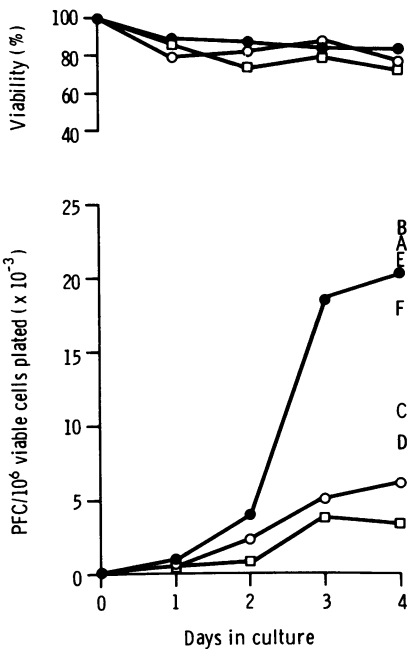

Figure 2. Inhibition of development of PFC against mouse brom RBC caused by culturing the cells with various RBC. The points are mean values for triplicate cultures. The minimum proportion of viable cells after 4 days in culture for the cultures A–E was 65%. ●, No RBC; ○, Normal mouse RBC; □, mouse brom RBC; A, horse RBC; B, horse brom RBC; C, sheep RBC; D, sheep brom RBC; E, rat RBC; F, rat brom RBC.

Table 1. The number of PFC against mouse brom RBC, and the proportion of SIg^+ cells and phagocytic cells before and after 4 days of culture of peritoneal cells

	Days in culture	
	Day 0	Day4
$PFC/10^6$ viable cells plated*	22 ± 10	$26{,}000 \pm 10{,}000$
% Viable	100	79
% Phagocytic	52	46
% SIg^+	20	19
SIg^+ and phagocytic	4	3

* The number of $PFC/10^6$ viable cells cultured represents the mean of triplicate cultures ± one standard deviation.

Table 2. The number of PFC against mouse brom RBC and the proportion of SIg^+ cells and phagocytic cells before and after 4 days of culture of peritoneal cells following depletion of phagocytic cells using adherence to plastic

	Peritoneal cell subpopulation	Days in culture: Day 0	Day 4
$PFC/10^6$ viable cells plated*	Control	200	12,500
	Non-adherent	200	17,000
	Adherent	150	7,000
% Viable	Control	100	36
	Non-adherent	100	36
	Adherent	100	40
% SIg^+	Control	19	20
	Non-adherent	37	37
	Adherent	6	6
% Phagocytic	Control	61	57
	Non-adherent	20	20
	Adherent	80	80

* The number of $PFC/10^6$ viable cells cultured represents the mean of triplicate cultures ± one standard deviation.

lower number of SIg^+ cells although again not on a *pro rata* basis (Table 3).

The peritoneal B-cell subpopulation was also enriched before culture using the method of adherence to antibody-coated dishes. Using this technique, there was a close parallel between the number of SIg^+ cells in the B-cell enriched or depleted populations and the number of PFC (Table 4). No significant change in the proportion of SIg^+ cells or the proportion of phagocytic cells was detected after 4 days in culture.

The fractionation of peritoneal cells using the antibody-coated sheep RBC rosetting technique of Parish *et al.* (1974) gave subpopulations enriched for B cells and depleted of B cells (Table 5). Neither of these subpopulations, however, gave the characteristic increase in PFC against mouse brom RBC during 4 days of *in vitro* culture (Table 5). It is likely that the B-cell enriched population was contaminated with antigen from sheep RBC and, as these cells inhibit the PFC against mouse brom RBC (Fig. 2), the poor response was not unexpected.

The treatment of peritoneal cells with anti-θ sera and complement before culture significantly increased the number of PFC against mouse brom RBC detected on the third and fourth day of culture (Table 6). The

Table 3. The number of PFC against mouse brom RBC and the proportion of SIg^+ cells and phagocytic cells after 4 days in culture in which depletion of phagocytic cells using adherence to plastic was performed before the assay for PFC

	% Viable	PFC/10^6 viable cells recovered*	% SIg^+	% Phagocytic
Control cultures	58	54,000 ± 11,500	21	50
Non-adherent	23	290,000 ± 53,000	35	28
Adherent	43	10,000 ± 2,000	8	66

* The number of PFC/10^6 viable cells recovered represents the mean of triplicate cultures ± one standard deviation.

Table 4. The number of PFC against mouse brom RBC and the proportion of SIg^+ cells before and after 4 days of culture of peritoneal cells following depletion or enrichment of B cells using antibody-coated petri dishes

	Peritoneal cell subpopulation	Days in culture: Day 0	Days in culture: Day 4
PFC/10^6 viable cells plated*	Control	25 ± 5	18,000 ± 2,000
	Non-adherent	5 ± 5	6,000 ± 600
	Adherent	35 ± 5	28,000 ± 5,700
% viable	Control	100	67
	Non-adherent	100	72
	Adherent	100	90
% phagocytic	Control	51	51
	Non-adherent	46	50
	Adherent	25	24
SIg^+	Control	26	24
	Non-adherent	7	7
	Adherent	43	40

* The number of PFC/10^6 viable cells cultured represents the mean of triplicate cultures ± one standard deviation.

three-fold increase on day 4 was greater than that expected because of the enrichment of B cells from 20 to 28% (Table 6). The PFC were shown to be IgM producers because the addition of an anti-mouse serum prevented plaque formation wheras anti-mouse γ serum had no effect.

The effects of T-cell depletion were investigated further by exposing the cells that did not form rosettes with antibody-coated sheep RBC (B-cell depleted) to anti-θ serum and complement. The anti-θ treated subpopulation was found to have a significantly increased response to mouse brom RBC in comparison with untreated B-cell-depleted populations or unfractionated peritoneal cell populations (Table 7). Although the number of SIg^+ cells increased only about two-fold, following anti-θ treatment, the number of PFC increased more than twenty-fold (Table 7).

Table 5. The number of PFC against mouse brom RBC and the proportion of SIg^+ cells and phagocytic cells before and during 4 days of culture of peritoneal cells following depletion or enrichment of B cells, using the antibody-coated sheep RBC rosetting technique

	Peritoneal cell subpopulation	Days in culture 0	2	3	4
PFC/10^6 viable cells plated*	Contol	100	1700	8000	18000
	Interface (B-cell depleted)	100	1800	3700	2100
	Pellet (B-cell enriched)	100	ND†	ND	1200
% Viable	Control	100	65	48	42
	Interface	100	62	53	33
	Pellet	100	ND	ND	33
% Phagocytic	Control	45	36	43	40
	Interface	45	45	33	33
	Pellet	24	ND	ND	22
% SIg^+	Control	19	20	18	15
	Interface	3	4	3	6
	Pellet	35	ND	ND	36

* The number of PFC/10^6 viable cells cultured represents the mean of triplicate culture ± one standard deviation.
† ND, not done.

Table 6. The number of PFC against mouse brom RBC, and the proportion of SIg^+ cells and phagocytic cells appearing during 4 days of *in vitro* culture after treating the peritoneal cells with anti-θ serum and complement

	Peritoneal cell subpopulation	Days in culture 0	1	2	3	4
PFC/10^6 viable cells plated*	Control cell culture	200 ± 50	200 ± 70	1400 ± 300	5500 ± 450	14,500 ± 2,000
	Anti-θ-treated cell culture	200 ± 50	200 ± 100	1600 ± 200	22,000 ± 2000	46,000 ± 6000
% Viable	Control	100	50	50	38	35
	Anti-θ-treated	100	50	43	39	33
% Phagocytic	Control	61	50	ND†	ND	57
	Anti-θ-treated	57	50	ND	ND	57
% SIg^+	Control	19	19	ND	ND	20
	Anti-θ-treated	30	30	ND	ND	28

* The number of PFC/10^6 viable cells cultured represents the mean of triplicate cultures ± one standard deviation.
† ND, not done.

Table 7. The number of PFC against mouse brom RBC and the proportion of SIg^+ cells and phagocytic cells before and after 4 days in culture after exposing the interface cells recovered from the antibody-coated sheep RBC rosetting technique to anti-θ serum and complement

	Subpopulation of peritoneal cells	Days in culture	
		Day 0	Day 4
PFC/10^6 viable cells plated*	Control cells	150 ± 20	$15{,}300 \pm 4{,}000$
	Interface cells (B-cell depleted)	130 ± 10	$2{,}100 \pm 350$
	Anti-θ-treated interface	60 ± 15	$48{,}000 \pm 8000$
% Viable	Control	100	72
	Interface	100	66
	Anti-θ-treated interface	100	33
% Phagocytic	Control	47	47
	Interface	45	45
	Anti-θ-treated interface	65	66
% $SIg \pm$	Control	22	27
	Interface	4	4
	Anti-θ-treated interface	11	8

* The number of PFC/10^6 viable cells cultured represents the mean of triplicate cultured $\pm$ one standard deviation.

DISCUSSION

Peritoneal cells from unimmunized mice develop, after a few days in culture, a high number of PFC directed against mouse brom RBC (Lord & Dutton, 1975a; Pages & Bussard, 1975; Steele & Cunningham, 1978). We have used various procedures to enrich or deplete the peritoneal cell population of B cells to test the possibility that some of the PFC developing were due to cells other than B cells. The results suggest that the high level of autoreactivity is due to antibody production by B cells. We have also shown that the high level of autoreactivity is regulated by T-cells and can be inhibited by culturing the peritoneal cells with syngeneic RBC.

Inhibition of the response by RBC was shown indirectly by removing RBC before culture, using ammonium chloride in concentrations that do not affect the viability or functional capacity of lymphocytes (Santoli & Trinehieri, 1977). This procedure significantly advanced the time at which increased numbers of PFC were detected and the number detected (Fig. 1). The proportion of cells remaining viable was high (Fig. 1). Further evidence that the development of large numbers of PFC against mouse brom RBC *in vitro* was inhibited by RBC was observed in peritoneal cell suspensions to which mouse RBC or sheep RBC were added before culture (Fig. 2). This was not simply due to a toxic effect of the RBC because equivalent numbers of horse RBC or rat RBC did not inhibit the response, and the proportion of viable cells cultured was high (Fig. 2). Both modified and unmodified RBC from sheep or mice inhibited the response. The specific inhibition of the response by sheep brom RBC or mouse brom RBC has been reported (Lord & Dutton, 1975b). Unlike the present study, however, Lord and Dutton did not observe inhibition with unmodified sheep RBC or mouse RBC. High cross-reactivity between mouse brom RBC and sheep brom RBC, and a lower level of cross-reactivity between sheep RBC and mouse brom RBC has been noted (Bussard *et al*, 1977). Further, antibodies against the 'new' antigens exposed by treating mouse RBC or sheep RBC with bromelain can be elicited in animals injected with unmodified RBC, suggesting that these 'new' antigens are buried in the membranes of unmodified RBC (Cunningham, 1976; Cox *et al*, 1977). Thus it seems possible that the inhibition caused by sheep or mouse RBC may be due to the breakdown of these RBC in culture. That unmodified

mouse RBC inhibit the response is supported by the increased number of PFC observed in cultures treated with ammonium chloride prior to culture (Fig. 1). The inability of horse or rat RBC to inhibit the response is likely to be due to a lack of the appropriate determinants for the anti-mouse brom RBC antibodies. In PFC assays, horse RBC have been shown to share few antigens with sheep RBC (Cox and Cross, unpublished data).

The possibility that the large increase in PFC against mouse brom RBC was caused by the replication of a particular cell subpopulation is not supported by the results. First, the increase was observed in cultures in which DNA synthesis was minimal (Lord & Dutton 1975a; Cox, Evans & Brooks, unpublished data). Second, the respective proportions of SIg^+ cells and phagocytic cells before culture were not significantly different from those observed after 4 days in culture. (Tables 1, 2, 4–7).

Various procedures were used to show that enrichment of a subpopulation for B-cells correlated with an increased number of PFC against mouse brom RBC whereas enrichment for phagocytic cells did not. This is illustrated best in the experiments where B cells were enriched or depleted by exposing peritoneal cell suspensions to antibody-coated petri dishes before culture (Table 4). In the B-cell enriched, B-cell depleted, and control cultures, it can be calculated that about 7–8% of the SIg^+ cells formed plaques against mouse brom RBC.

That a very high proportion of the B cells formed plaques against brom mouse RBC was shown by selectively assaying non-adherent cells after 4 days in culture (Table 3). In these experiments, the number of PFC detected was expressed with respect to the number of viable cells recovered rather than cultured originally because the subpopulations were manipulated separately after the culture period. The data presented in Table 3 do not support the idea that phagocytic cells are forming spurious plaques during the assay because the subpopulation enriched for phagocytic cells had a lower number of PFC than the subpopulation depleted of phagocytic cells. The requirement of cells to undergo protein synthesis to form plaques (Lord & Dutton, 1975a) and the fact that PFC can be micromanipulated to form more than one plaque (Bussard *et al*, 1977) suggest that B cells are actually secreting the antibodies that lyse brom mouse RBC.

The sheep-RBC-rosetting technique of Parish *et al.* (1974) gave a subpopulation enriched for B cells that did not form a high number of PFC against mouse brom after *in vitro* culture (Table 5). In view of the inhibitory activity of sheep RBC on these responses (Fig. 2), it seems that the most likely explanation for the low response is that B cells became associated with sheep RBC antigens and debris during the separation procedure. The low number of PFC detected in the interface subpopulation may be due to enrichment for T-cell suppressors. T cells are known to be concentrated at the interface of the gradient (Parish *et al.* 1974). Thus the increase in suppressor cells and the decrease in B cells may have facilitated effective regulation of those B cells during the culture period. This view is supported by the observation that treatment of the B-cell depleted cells with anti-θ serum and complement before culture caused a big increase in the number of PFC against mouse brom RBC (Table 7).

The fact that antibody production against antigens on mouse brom RBC antigens may be regulated by T cells, as suggested by Cunningham (1976) from *in vivo* studies, was supported by the observation that removal of T cells using anti-θ serum and complement prior to culture caused a significant increase in the number of PFC detected (Tables 6 and 7). It is unlikely that the increases observed were due to LPS contamination of the sera because control cultures, exposed to the complement source did not show the increases. In the treated cultures the increased response did not occur until day 3 which is not typical of the rapidly developing response by serous cavity cells caused by LPS (Steele & Cunningham, 1978). Further, polyclonal activation by LPS would be expected to cause a proportionately smaller increase in the numbers of PFC in cultures depleted of B cells (e.g. compare Tables 7 and 6), which did not occur.

The B-cell enriched subpopulation obtained by allowing phagocytic cells to adhere to plastic prior to culture produced a greater number of PFC than the control cultures (Table 2), although the increase in PFC was not in proportion to the increase in B-cells. In other systems it has been noted that macrophages produce factors that augment lymphocyte activity *in vitro* (Calderon, Kiely, Lefko & Unanne, 1975). Thus the enrichment in B cells and depletion of macrophages may not have generated culture conditions as favourable for B cells as those in control cultures. The proportion of SIg^+ cells in the adherent population forming plaques against mouse brom RBC was greater than the proportion in control cultures and non-adherent cultures (Table 2). Again the increased proportions of macrophages may have enhanced the

activity of the remaining B cells. In addition, the effect may be due to the removal of a non-adherent suppressor cell population during the initial 4 h culture.

Enrichment or depletion of the cultures of phagocytic cells did not correlate with changes in the numbers of PFC against br mouse RBC detected (Table 2). This suggests that the plaques are not spurious simply due to uptake of antibodies by macrophages during culture and release during the plaque assay. The lower proportion of viable cells after 4 days in culture is probably due to the damage to cells caused by the manipulations used to separate non-adherent and adherent cells prior to culture.

The experiments presented here, and elsewhere (Steele & Cunningham, 1978) suggest that a high proportion, if not all, B cells from the peritoneal cavity secret IgM antibodies that can lyse mouse brom RBC after a few days in culture. This autoreactivity, which is present in low levels *in vivo*, appears to be regulated by T-cells and/or antigens on mouse brom RBC. The high level of autoreactivity that develops *in vitro* apparently involving all B cells of the peritoneal cavity, is difficult to explain with the present rules of immunology although recently, it was shown that a high proportion of IgM-PFC produced autoantibodies (Dresser, 1978).

A possible explanation is that B cells lysing mouse brom RBC are concentrated in the peritoneal cavity because they are directed against antigens on the microbes of gut flora. If these microbes gain entry to the peritoneal cavity, primed but suppressed B cells are released from suppression and produce antibodies to eliminate the invaders. There is a lot of evidence to show that some antigenic determinants are common to both mammalian cells and microbes (Asherson, 1968). A problem, however, is that the role of antigen would be to stimulate the response rather than suppress as is the case with mouse brom RBC (Lord & Dutton, 1975a, Fig. 2). Also, it is unlikely that the range of antigenic determinants on various gut microbes would be present on mouse brom RBC.

Another explanation is that unstimulated B cells, of various specificities, are normally prevented from secreting autoantibodies apart from a small proportion of cells, by suppressor T cells. During *in vitro* culture, suppressor activity wanes and the B cells start secreting antibodies of various specificities for antigens. It is speculated that each antibody has in addition to its unique combining sites for antigens, another reactive site that can bind to sheep or mouse brom RBC and to unmodified sheep RBC leading to activation of complement and lysis. It is not clear why these sites are not present on rat or horse brom RBC.

An obvious objection is that mice injected with sheep RBC contain many spleen cells secreting IgM antibodies against sheep RBC but do not contain significantly increased levels of PFC to mouse brom RBC (Cox *et al.*, 1977). It is thus necessary to speculate further that IgM antibodies secreted in response to antigenic stimulation are of a slightly different molecular nature to those secreted spontaneously either *in vivo* or after *in vitro* culture. The suppression of the response *in vitro* by mouse brom RBC may be a form of paralysis where any B cell, that has escaped T-cell control and displays these unusual IgM receptors with a combining site for specific antigen and another site for mouse brom RBC encounters a large amount of RBC and is paralysed unless it changes the nature of its IgM receptor. The specific blocking of PFC against mouse brom RBC by specific antigen (DeHeer & Edgington, 1974) or by mouse RBC stromata (Bussard *et al.*, 1977) is not incompatible with the idea that the PFC were releasing antibodies interacting with mouse brom RBC at a site independent of their specific combining site for antigenic determinants. It seems that these ideas could be tested easily by comparing the molecular nature of 'spontaneously' produced IgM antibodies with those produced during active immunization.

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NOTE ADDED TO PROOF

Pages, J.M. & Bussard, A.E. (*Cell. Immunol.* **41,** 188, 1978) have established hybridomas secreting antibodies against mouse brain RBC.